Molecular analysis of a gene encoding a serum-resistance-associated 76 kDa surface antigen of *Haemophilus somnus*

S. P. COLE,^{1,2*} D. G. GUINEY² and L. B. CORBEIL¹

Departments of Pathology¹ and Medicine², 8416, University of California San Diego Medical Center, 200 W. Arbor Dr., San Diego, California 92103-8416, USA

(Received 9 February 1993; revised 17 May 1993; accepted 25 May 1993)

Haemophilus somnus is a Gram-negative bacterial bovine pathogen which can cause disease or be carried asymptotically. We previously showed that four serum-sensitive isolates from asymptomatic carriers lacked a 13.4 kb sequence of chromosomal DNA that was present in two virulent serum-resistant strains. We have since sequenced 5 kb of the 13.4 kb fragment from a serum-resistant strain, which contained an open reading frame (ORF) of at least 4.5 kb. From Western blot analysis, the ORF was shown to encode a 76 kDa protein (p76) that co-migrated with a 76 kDa *H. somnus* surface protein. Both the recombinant and natural p76 reacted with convalescent-phase serum from a cow in an experimental *H. somnus* abortion study. The translational start site for p76 was identified by deletion analysis of subclones of the 5 kb cloned sequence. The 4.5 kb ORF contained 1.2 kb tandem direct repeats (DRs), with 65% identity between the two repeats at the protein level. The 5' DR (DR1) included the start site for the 76 kDa protein, and DR2 had a flanking inverted repeat, suggestive of an insertion-sequence-like element.

Introduction

The bovine pathogen *Haemophilus somnus* is the causative agent of a wide range of both septicaemic and nonsepticaemic syndromes including pneumonia, infertility and the sequelae of septicaemia: abortion, meningoencephalitis, arthritis and myocarditis (for reviews, see Harris & Janzen, 1989; Humphrey & Stephens, 1983; Kwiecien & Little, 1991). The infection may be sexually transmitted also (Kwiecien & Little, 1991), since a genital asymptomatic carrier state is common. Most normal bulls are preputial carriers and some normal cows are vaginal carriers (Kwiecien & Little, 1991). Others have shown that genital isolates from asymptomatic carriers are avirulent or less virulent than isolates from diseased animals (Groom *et al.*, 1988; Humphrey, 1982; Humphrey & Stephens, 1983; Inzana & Todd, 1992). Our own studies (Corbeil *et al.*, 1985) demonstrated that several preputial isolates from asymptomatic carriers were serum-sensitive, whereas all isolates

that could be causally associated with disease were serum-resistant. The resistance to killing by fresh bovine serum was associated with the presence of 270 and 41 kDa outer-membrane proteins (OMPs) (Widders *et al.*, 1989) and/or lipooligosaccharide with multiple bands on silver-stained gels (Inzana & Todd, 1992). The 270 and 41 kDa OMPs (p270 and p41) were both immunoglobulin-binding proteins (IgBPs; Yarnall *et al.*, 1988*a, b*).

A cosmid library from a virulent, serum-resistant strain produced a clone that expressed two additional but related serum-resistance-associated proteins at 76 and 120 kDa (p76 and p120) that reacted with antiserum to the 270 kDa IgBP (Corbeil *et al.*, 1988; Cole *et al.*, 1992). The proteins were visualized by Western blot, reacting with protective convalescent serum or antiserum to the 270 kDa IgBP. Later, the genes for these two proteins were localized to a 13.4 kb sequence (Cole *et al.*, 1992). Southern blots showed that two virulent, serum-resistant isolates contained this sequence, whereas four serum-sensitive strains from asymptomatic carriers lacked this segment of DNA (Cole *et al.*, 1992). Additional Southern blots with probes constructed from fragments of each of these two genes showed no cross-hybridization, suggesting that p76 and p120 had no significant DNA homology. Because both proteins reacted with polyclonal antibody to p270, the lack of

* Author for correspondence. Tel. (619) 543 6146; fax (619) 543 6614.

Abbreviation: DR, direct repeat.

The GenBank accession number for the sequence of the entire 4.5 kb ORF reported in this paper is L10282.

homology between the two indicated that p270 may be a polymer of p76 and p120.

In this paper, we present the sequence of the gene for the 76 kDa protein. By analysis of Western blots of proteins expressed by subclones, we determined the probable start codon of the p76 gene. We also report the existence of a large insertion-sequence-like direct repeat within this gene and upstream regions of *H. somnus* chromosomal DNA.

Methods

Bacterial strains and plasmids. *H. somnus* isolates were grown in brain heart infusion medium (Difco) supplemented with 0.1% Trizma base and 0.001% thiamin monophosphate (BHITT), as described previously (Inzana & Corbeil, 1987). Solid medium was BHITT agar plus 5–10% (v/v) calf blood, as described (Cole *et al.*, 1992). Serum-resistant strain 2336, from which all the subclones were derived, was isolated from a pneumonic calf and has been used to reproduce *H. somnus* pneumonia (Gogolewski *et al.*, 1987). The subclones in this paper were derived from cosmid pHS1 (Corbeil *et al.*, 1988), containing a 35 kb insert of *H. somnus* DNA in *E. coli* DH1. Transformants in *E. coli* DH5 α were grown in LB medium (Sambrook *et al.*, 1989) supplemented with ampicillin at 100 μ g ml⁻¹.

Recombinant DNA techniques and subclone construction. DNA isolation for construction of plasmids and subclone analysis was by the boiling method (Holmes & Quigley, 1981). All other DNA manipulations were carried out using standard protocols (Sambrook *et al.*, 1989). Plasmids pHS139 and a derivative, pHS138, were constructed as previously described (Cole *et al.*, 1992). Briefly, pHS139 consisted of a 5 kb *Xba*I–*Pvu*II insert from pHS1 in pUC19, while the derivative pHS138 contained a 3 kb *Hpa*I–*Pvu*II insert cloned in pUC18. Deletion derivatives of pHS138 were made using appropriate restriction enzymes, followed by religation of the plasmid. Specifically, pHS176 was constructed by cloning the *Pvu*I–*Pst*I fragment from pHS138 into the *Sma*I–*Pst*I site of pUC18. This resulted in the p76 gene lying just downstream of the pUC18 *lacZ* start site. Plasmid pHS182 was made by treating pHS176 with *Eco*RI, filling in the ends with the large fragment (Klenow) of DNA polymerase, then religating the plasmid. This treatment placed the p76 gene out of frame with respect to the *lacZ* ATG start site. Finally, plasmid pHS171 was prepared by digestion of pHS138 with *Bst*BI and *Eco*RI, followed by religation. All of these plasmids were tested by restriction analysis and gave the appropriate sized fragments.

SDS-PAGE and Western blots. Western blots were from 8% SDS-polyacrylamide gels of whole cell lysates. The cells were prepared for loading in sample buffer as previously described (Cole *et al.*, 1992). Briefly, cultures were adjusted to an optical density of 0.4 at 600 nm, and then a 1.5 ml vol. was pelleted and resuspended in 100 μ l of sample buffer prior to loading 3 μ l in the wells. After electrophoresis, proteins were electrotransferred to nitrocellulose (Schleicher and Schuell) as previously described (Cole *et al.*, 1992). Filter blots were made from the gel, and reacted with a 1:2000 dilution of serum P3, as before (Corbeil *et al.*, 1988; Cole *et al.*, 1992). Serum P3 was convalescent phase serum taken from a cow in an experimental abortion study using a serum-resistant strain of *H. somnus* (Widders *et al.*, 1986). This serum reacts with the 270 kDa, 120 and 76 kDa antigens as well as other *H. somnus* antigens not expressed in the *E. coli* recombinants reported in this study (Corbeil *et al.*, 1988; Cole *et al.*, 1992). Specific antiserum to p270 was raised in rabbits by inoculation of the gel-purified antigen as previously described (Yarnall *et al.*, 1988b). The antiserum reacts only with *H.*

somnus antigens p270, p120 and p76, even though there is no significant DNA homology between p76 and p120 (Cole *et al.*, 1992). This suggested that p76 and p120 may be subunits of p270 (Cole *et al.*, 1992). Gamma-Bind G-horse radish peroxidase conjugate (Genex Corp.) at a 1:2000 dilution was used to detect antigen-antibody reactions, with the addition of 4-chloro-1-naphthol and hydrogen peroxide.

Sequencing strategy. Double-stranded sequencing was carried out using dideoxynucleotide termination reactions with T7 DNA polymerase in a multiwell microtitre plate system (Amersham). To resolve some ambiguous sequences, Taq polymerase (Promega) was used at higher temperatures. Clones and subclones were sequenced using both forward and reverse universal pUC primers and overlapping areas with synthetic primers (from Molecular Biology Core Facility, University of California, San Diego, CA).

Data analysis. DNA was analysed using DNA Strider (shareware) and MacVector software (International Biotechnologies Inc.).

Results and Discussion

Cosmid pHS1 contained a 35 kb insert from *H. somnus* that expressed a 76 kDa antigen and low levels of a group of *H. somnus* antigens centred at 120 kDa on SDS-PAGE gels when expressed in *E. coli* (Corbeil *et al.*, 1988). A 12 kb section of the cosmid insert was subcloned into the vector pUC19 (creating pHS134). This plasmid expressed high levels of the 120 kDa group of proteins, as well as the p76 and lower molecular mass proteins (Cole *et al.*, 1992). Further subcloning of this insert into pHS139 and pHS140 on pUC-based vectors resulted in the expression of 120 kDa proteins from pHS140, while pHS139 produced large amounts of p76, as well as lower molecular mass proteins (Cole *et al.*, 1992). Expression was determined by Western blots using cell lysates of the subclones reacted with convalescent phase serum or polyclonal antiserum to p270, which reacts with both p76 and p120 (Cole *et al.*, 1992). The 5 kb *Xba*I–*Pvu*II insert in plasmid pHS139 was subcloned in pUC-based vectors in either orientation with respect to the *lacZ* promoter to give pHS138 and pHS137. Both of these plasmids contained 3 kb *Hpa*I–*Pvu*II inserts. For pHS138, expression from the *lacZ* promoter resulted in large quantities of a 76 kDa protein, as well as several lower molecular mass antigens, all of which reacted with antiserum to the 270 kDa IgBP. Plasmid pHS137 produced minimal amounts of the same proteins. The same bands were observed with the larger pHS139, although the 5 kb insert was in the opposite orientation with respect to the *lacZ* promoter from pHS138. This indicates that expression in *E. coli* of pHS139 was from a promoter originating from *H. somnus*, but that high-level expression in pHS138 was from the vector *lacZ* promoter.

DNA sequence analysis of the 5 kb *Xba*I–*Pvu*II insert from pHS139 revealed a single 4.5 kb ORF, with no other ORF present in any other frame. The ORF may

```

1681      *           *           *           *           *
ATA CAA AAA GGA AGT GCG GTA GAT GAT CTC AAT GCA CAA ACT TTA GCG ATT ACA GAA GCT

1741      *           *           *           *           *
TTA GCA GAT ACG TCA AAA ACA ATT CAA CGT AAT CCA AAA TTG AAA GAA GAA GTT CAA GGT

1801      *           *           *           HpaI           * A 76 kDa *
GCG ATT GAA GAA TTT TTG AAA AGT AGT CAG GGC AAA GAG TTA ACT GTA GAG ATG ATC GAA
                                         M I E

1861      *           *           *           PvuI           *           *
AAG TTA AAT CAT GGA TTA CGT CCT GAT GAA GGT AGC GAT CGT TTG CTT TAC AAA AAG GAA
K L N H G L R P D E G S D R L L Y K K E

1921      *           *           *           *           *           *
AAT TTA ACT AAA GAA AAT GCG GTA TTT TCC AGT CCG CAA GCA TCA AAA ATT CAG TTA AAT
N L T K E N A V F S S P Q A S K I Q L N

1981      *           *           *           *           *           *
GAA ACA GTG GAC TTT ATT AAC CAA GCA ATC AAG CAA AAT GTG GAA CCA AGT GTT TTA GCG
E T V D F I N Q A I K Q N V E P S V L A

2041      *           *           *           *           *           B *
GGG TTG GTT TAT CAG CGA TTA ATT GCG TAC CAT CCG TTC GCA GAA GGG AAT GGT CGA ATG
G L V Y Q R L I A Y H P F A E G N G R M

2101      *           *           *           *           *           *
GCT CGA GTT GTA GTA AAT AAA ATT TTA CTT GAT GCA GGT TAT CCG CCG TTT ACT AAA TTC
A R V V V N K I L L D A G Y P P F T K F
2161      *           *           *           *           *           *
AGT TCT GAA TTT GAA ACG CAG ATC ATT CCG CAA ACA AAA GCT ACG GCT AAA TCA GCA ACA
S S E F E T Q I I P Q T K A T A K S A T

2221      *           *           *           *           *           *
AGT GCG GAA GTT GTG AAA GAG TTT TTG ACT GAA TTA GGT AAA AAA TCA TCT CCG CAA GAG
S A E V V K E F L T E L G K K S S P Q E

2281      *           *           *           *           *           *
GGA GGT GCG AAT AAT CAA AAC GGA CAA GCG ACT TCG CCT GTT ACG TTG AAG TCT AAA GAT
G G A N N Q N G Q A T S P V T L K S K D

2341      *           *           *           *           *           *
GTT AGT GAA GTC GAA AAC ACA CAA TCC GCA GAC AGT TTG ACC ATT AAA CAG CCG GAA CAA
V S E V E N T Q S A D S L T I K Q P E Q

2401      *           *           *           *           *           *
GGT AAA GCA GGT GGT CAG TTA CCA TCT GTT CCG AAA GTG GAG ACT TCT GTA AAT GAA GTT
G K A G G Q L P S V P K V E T S V N E V
----->

2461      *           *           *           *           *           *
GCA CCG TTG TCA TCT GTA CCA GCA GAA CTA AAA GAT GCT GCT GGA GGA AAT AAA AAA GCG
A P L S S V P A E L K D A A G G N K K A

2521      *           *           *           *           *           *
GCA GAG AAA TCA GAG GGT GCT ACG GGT GTA GAA AAA GAA AAA ACC ACA CTT TTC CAA CGA
A E K S E G A T G V E K E K T T L F Q R

2581      *           *           *           *           *           *
GTG AAA CAA TTT TTC ACC GGA AGT AAG AGC GGT GCG AAA CCT GTA GCG GGA GAT GAG ACA
V K Q F F T G S K S G A K P V A G D E T

2641      *           *           *           *           *           *
GCG AAT AAA GTC AAT TAT CAA GAT TTG GAA GAT AAT TTG AAC TTA AAA GGA TTA ATT TCT
A N K V N Y Q D L E D N L N L K G L I S

```

```

2701      *           *           *           *           *
TTA GAA GAT GAT CGA AAT GCT AAT TTT GAA AGT AAT GTA TTG AAA AAT GAG AAA TTT TTA
L   E   D   D   R   N   A   N   F   E   S   N   V   L   K   N   E   K   F   L

2761      *           *           *           *           *           C           *
GAT GAA GCA AGA GAG ATT TCG AAG AAA TCA ATT CCT GAA GCG ACA GTT AAG CAA ATG TCT
D   E   A   R   E   I   S   K   K   S   I   P   E   A   T   V   K   Q   M   S

2821      *           *           *           *           *           *           *
CAT TTA CCT GAA TTT GAT GAT ATT CTC ACC GAG GGA GCT AAG AAA GTA GAA AGT CGT ATT
H   L   P   E   F   D   D   I   L   T   E   G   A   K   K   V   E   S   R   I

2881      *           *           *           *           *           *           *
AAT AAG GCA ATC ACA TTC CGC CCT TCT GTT GAG GAG TTT TCA GAA ATT CAA GAT TTG GTG
N   K   A   I   T   F   R   P   S   V   E   E   F   S   E   I   Q   D   L   V

2941      *           *           *           *           *           *           *
AAA ACG TTA CCG AAA ACA AAG GTT ATA GAG GAT CTT TCA ACA AAA ACA AAT GAA ATC ACA
K   T   L   P   K   T   K   V   I   E   D   L   S   T   K   T   N   E   I   T

3001      *           *           *           *           *           *           *
GAA GCT TTA GCT GCG ACA TCG AAA ACC ATT CAA CGT ACA CCG GAG TTG AAA GAA CAG TTG
E   A   L   A   A   T   S   K   T   I   Q   R   T   P   E   L   K   E   Q   L

3061      *           *           *           *           *           *           *
AAG ACA GCA ATA GAG GAT TTC TTA CAA AAC AGT CAA GGC AAA CCT TTG ACA GTG CAG ATG
K   T   A   I   E   D   F   L   Q   N   S   Q   G   K   P   L   T   V   Q   M

3121      *           *           *           *           *           *           *
ATC GAG AAT CTT AAT CAC GGA TTA CGT CCG GAT GAG GGA GAA GGT CGT TTA CTT TAT AAA
I   E   N   L   N   H   G   L   R   P   D   E   G   E   G   R   L   L   Y   K

3181      *           *           *           *           *           *           *
AAA GAG AAT TTA ACC AAA GAA AAT GCG GTA TTT TCT AGT CCC GAA GCG GCA AAA ATT CAA
K   E   N   L   T   K   E   N   A   V   F   S   S   P   E   A   A   K   I   Q

3241      *           *           *           *           *           *           *
TTA GCG GAA ACG GTT GAT TTT ATC AAT CGA GCG AAA AAT GAA GGG ATT GAG CCG AGT GTG
L   A   E   T   V   D   F   I   N   R   A   K   N   E   G   I   E   P   S   V

3301      *           *           *           *           *           *           *
GTT GGG GCA TTA GTT TAT CAG CGA TTG ATT GCT TAT CAC CCA TTT GCA GAA GGT AAT GGA
V   G   A   L   V   Y   Q   R   L   I   A   Y   H   P   F   A   E   G   N   G

3361      *           *           *           *           *           *           *
CGT ATG GCG AGA GTC ATA GTA AAT AAA ATT TTA CTT GAT GCA GGT TAT CCG GCA TTT ACC
R   M   A   R   V   I   V   N   K   I   L   L   D   A   G   Y   P   A   F   T

3421      *           *           *           *           *           *           *
AAA TTT AGT GAT GAG TTT GAA CCG CAG ATT ATT CCT CAA ACG AAA GCA TCA ACT AAA TCC
K   F   S   D   E   F   E   P   Q   I   I   P   Q   T   K   A   S   T   K   S

3481      *           *           *           *           *           *           *
GCA ACG AGC AGT GAA GTG GTA GTT GAG TTT TTA AAA GAG TTG GCA AAA AAA GGA AGC AAG
A   T   S   S   E   V   V   V   E   F   L   K   E   L   A   K   K   G   S   K

3541      *           *           *           *           *           *           *
GAA GAT AAC GAG CAG AAT TTA GAA AAA ACT GAC CGC ACT TCT ACG GAC TTG ACA GAA AGT
E   D   N   E   Q   N   L   E   K   T   D   R   T   S   T   D   L   T   E   S

3601      *           *           *           *           *           *           *
GCG GTA GAA AAT TCG GCT GCT TTG AGT TCC GGA ACA GTT AGA TCT GCG ACA GTT TCT GAA
A   V   E   N   S   A   A   L   S   S   G   T   V   R   S   A   T   V   S   E

<-----

3661      *           *           *           *           *           *           *
ACA GTT ACT GAA ACG GAA CAG GCA AAA GCG AAA CCA GTT AGT GAT TTG GTG AGC AGT AAA
T   V   T   E   T   E   Q   A   K   A   K   P   V   S   D   L   V   S   S   K

```

```

3721      *      *      *      *      *
GAT TTA GTG GAA CAG CAA CGC ACG GTA TTG CAA CGC ATT CAG GAT CAA TTT CAG CCT TTG
D   L   V   E   Q   Q   R   T   V   L   Q   R   I   Q   D   Q   F   Q   P   L

3781      *      *      *      *      *
AAA GTG AAA AGC AAA ATT GAT GCT GTA CGT TCT TCC GTT GAA GAG TTT GGT GGA GAA GTA
K   V   K   S   K   I   D   A   V   R   S   S   V   E   E   F   G   G   E   V

3841      *      *      *      *      *
AGC TTT AAG TTT GCC CAA TCT AAA GGA GAA GTT TAC AAG GAA ATT GTA AAA CAC ATT GAA
S   F   K   F   A   Q   S   K   G   E   V   Y   K   E   I   V   K   H   I   E

3901      *      *      *      *      *
ACA CAA AAT GGT GTG TGT GAA TCA ACT TGT GCA CAT TGG ATT GCA AAA AAT GTT AAT CCA
T   Q   N   G   V   C   E   S   T   C   A   H   W   I   A   K   N   V   N   P

3961      *      *      *      *      *
ACC GAC GAG AAT TTC TTT AAT ACC CTT TAT GAA GGT GGT AAA AAA GGA CAC TTG AAA AAA
T   D   E   N   F   F   N   T   L   Y   E   G   G   K   K   G   H   L   K   K

4021      *      *      *      *      *
GAA ACT ATT GAT TCT ATT AAA AAA CTT CAA ACT GAG TTT ATT AAT AGC GGT AGT GCG ACT
E   T   I   D   S   I   K   K   L   Q   T   E   F   I   N   S   G   S   A   T

4081      *      *      *      *      *
CAA CAA TTC AAA TTG ACA GAT AGT TGG TTG CAA GAG CAG GGT GTT GTT CCG AAA GAG AAA
Q   Q   F   K   L   T   D   S   W   L   Q   E   Q   G   V   V   P   K   E   K

4141      *      *      *      *      *
AAA GTA GCA GAT TTT GTT CGT CGT GAT GAA GTA TCA GGA ACT GTA TCA AAA AAT GAT GTA
K   V   A   D   F   V   R   R   D   E   V   S   G   T   V   S   K   N   D   V

4201      *      *      *      *      *
TCC TCA TTA GTA AAA GCG ATT TTG GAT ACA GGC GAT GAT ACT GCC GGT GTT AAG AAA ATC
S   S   L   V   K   A   I   L   D   T   G   D   D   T   A   G   V   K   K   I

4261      *      *      *      *      *
TCC ATT AAT CTT GAG GGA GGA TCT CAC ACT GTA TCT GCG GCG GTT GAC GGC TCA AAA GTT
S   I   N   L   E   G   G   S   H   T   V   S   A   A   V   D   G   S   K   V

4321      *      *      *      *      *
ACT TTC TTT GAT CCG AAT TTT GGT GAA ATG ACA TTT CCA ACT CAT CAG CAG TTT GAG AAT
T   F   F   D   P   N   F   G   E   M   T   F   P   T   H   Q   Q   F   E   N

4381      *      *      *      *      *
TGG TTG AAG AAT GCA TTT TGG CAA AAA AGT GGC TAC GCT GGA AAG CAA GAA GGA AGA CGT
W   L   K   N   A   F   W   Q   K   S   G   Y   A   G   K   Q   E   G   R   R

4421      *      *      *
TTC TTT AAT GTG GTC AAC TAC AAA AAA AAT AAT TGA
F   F   N   V   V   N   Y   K   K   N   N   *

```

Fig. 1. p76 gene sequence and upstream DNA region. The two direct repeats (DRs) are italicized and underlined. Since the p76 gene starts translation in the middle of DR1, only that portion of DR1 that codes for p76 and some of the upstream sequence is shown. Arrows indicate inverted repeats flanking DR2. Sequence locations are in base pairs, originating from the *Xba*I site of pHS139. *Hpa*I and *Pvu*I sites are located in the plasmid insert (Figs 2 and 4). Possible ATG start sites, also indicated in Fig. 4, are labelled A, B and C.

extend further upstream from the sequence reported in this paper, since no stop codon was encountered. The GC content of the entire 4.5 kb ORF is 39.3%, which is consistent with the published sequence of the *H. somnus* ribosomal RNA gene and flanking two ORFs (38.7%) (Theisen & Potter, 1992), the 40 kDa *H. somnus*

lipoprotein (36.2%) (Theisen *et al.*, 1992), and the previously determined value (37.3% $\pm$ 0.2) for total *H. somnus* DNA (Bailie *et al.*, 1973). The sequence for the p76 coding region is presented in Fig. 1. The derived amino acid sequence of p76 was analysed for hydrophilicity using the algorithm of Kyte & Doolittle (1982)

Fig. 2. The 4.5 kb ORF of pHS139, showing the two DRs and the p76 gene (arrow). The translational start site for the p76 gene lies between the *HpaI* and *PvuI* restriction sites (see Fig. 4). The three repeated methionines within each DR are indicated by vertical bars above the horizontal line representing the sequence.

with a window size of seven residues (data not shown). The peptide was predicted to be highly hydrophilic, with no long hydrophobic regions characteristic of a membrane-spanning protein, and did not contain an obvious signal sequence (Von Heijne, 1983). Our earlier studies showed the *H. somnus* 76 kDa surface protein to be shed in the soluble fraction of culture supernatant (S. A. Kania, M. Yarnall & L. B. Corbeil, unpublished

data). These observations suggest that p76 may be a peripheral membrane protein or a surface protein rather than an integral membrane protein.

A striking feature of the large ORF is the presence of two large, approximately 1.2 kb direct repeats (DRs – Fig. 2), one of which (DR2) is flanked by an imperfect inverted repeat of 11 bp, with only one mismatch (Fig. 1). Although the start codon of the p76 gene is in the middle of DR1 (see evidence below), the entire DR1 was translated in Fig. 3 in order to show the alignment of DR1 with DR2. The most homologous portion of the DRs is the centre, while the ends of the DRs tend to be more divergent (Fig. 3). Also, the two DR sequences are much more homologous at the protein level (64% identity over 404 amino acids), than at the DNA level (31% identity).

Because of the inverted repeats flanking DR2 and the size similarity to other insertion sequences, it was of interest to explore the possibility that the DRs represent a foreign inserted sequence. Therefore, codon usage of the translated region of DR2 was compared (data not shown) to that of the C-terminus of the p76 ORF as well

DR1	<i>SVALGTPKSAV-AESNRNNGNQKLQSEGAEGVSPKTKSEDKSWFAKVKDFFFAKSNKSQA</i>
DR2	<i>SVNEVAPLSSVPAELKDAAGGNKAA-EKSEGATGVEK-EKTTLFORVKOFFTG-S-KSGA</i>
DR1	<i>KEAKSEQETVSKPNYDSLEDDLNLKNLLALEDKRGSSFEENVLKNPEFLAEAREIAKKYIP</i>
DR2	<i>KPVAG-DETANKVNYODLEDNLNLKGLISLEDDRNANFESNVLKNEKFLDEAREISKKSIP</i>
DR1	<i>EATIKQMGNSPFEDEILTEGAKKVEKRINDALTFKPSVDEFNEIQGLVKNIQKGSVDDLN</i>
DR2	<i>EATVKOMSHLPEFDDILTEGAKKVESRINKAITFRPSVEEFSEIODLVKTLPKTKVIEDLS</i>
	>>p76 start>>
DR1	<i>AQTLAITEALADTSKTIQRNPKLKEEVQGAIEEFLKSSQGKELTVEMIEKLNHGLRPDEGS</i>
DR2	<i>TKTNEITEALAATSKTIORTPELKEQLKTAIEDFLONSOGKPLTVOMIENLNHGLRPDEGE</i>
DR1	<i>DRLLYKKENLTKENAVFSSPOASKIOLNETVDFINOAIKONVEPSVLAGLVYORLIAYHPF</i>
DR2	<i>GRLLYKKENLTKENAVFSSPEAAKIOLAETVDFINRAKNEGIEPSVVGALVYORLIAYHPF</i>
DR1	<i>AEGNGRMARVVVNKILLDAGYPPFTKFSSEFETOIIPOTKATAKSATSAEVLVKEFLTELGK</i>
DR2	<i>AEGNGRMARVIVVNKILLDAGYPAFTKFSDFEFOIIPOTKASTKSATSSSEVVVEFLKELAK</i>
DR1	<i>KSSPOEGGANNONGOATSPVTLKSKDVSEVENTOSAD</i>
DR2	<i>KGSK-ED--NEONLEKTDR-TSTDLTESAVENSAALS</i>

Fig. 3. Alignment of the DNA and translated protein sequences of the two DRs. The portion of DR1 upstream of the translational start site for p76 is indicated in italics. The coding sequence for p76 is underlined. Vertical lines represent identities at the protein level.

Fig. 4. *Left*. Inserts from subclones of pHS139, in the vectors pUC18 or 19, used in deletion analysis. The restriction map of the insert from pHS139 is indicated at the top. The ORF, with stop codons and ATG codons as in Fig. 1, is shown below. The ATG codons discussed in the text are indicated A, B and C. Possible translational fusions with the start ATG codon of *lacZ* are indicated. *Right*. Western blot of deletion derivatives reacted with P3 convalescent-phase serum. Molecular masses are indicated in kDa.

as four other published *H. somnus* ORFs (Theisen & Potter, 1992; Theisen *et al.*, 1992). For five amino acids [leucine (UUG), valine, threonine, alanine and glycine] guanine was preferred more as the third base of the codon for DR2 as compared to the other four *H. somnus* ORFs. There was a concomitant decrease in the use of adenine as the third base for these amino acids, with the exception of glycine. The most notable differences between DR2 and the C-terminus of the p76 ORF were with the codon usage of leucine, isoleucine, proline and tyrosine. For the rest of the 20 main amino acids, there was no significant difference between DR2 and these other *H. somnus* sequences. Since DR2 varied in its codon usage from other *H. somnus* sequences, DR2 codon usage was further compared to that of the closely related bovine pathogen *Pasteurella haemolytica* (Lo, 1992), as well as to *E. coli* (Sharp *et al.*, 1988), host of many other insertion sequences. In both cases, the codon usage differed significantly from DR2 (data not shown); thus it is improbable that the DRs originated from either of these other organisms. Nevertheless, since the sequence of DR1 differs substantially from DR2, it appears likely that if insertion and duplication events occurred, they happened long ago.

There have been numerous instances of insertion

elements affecting the expression of genes involved in virulence, including IS1 inserts in the *virF* gene (an invasion-positive regulator) of *Shigella flexneri* 2a (Mills *et al.*, 1992), a mosaic of multiple insertion-sequence-like elements upstream of the *Pseudomonas aeruginosa* exotoxin A gene (Pritchard & Vasil, 1990), and three tandem insertion-sequence-like elements upstream of variable antigen genes in *Borrelia hermsii* (Barbour *et al.*, 1991). Thus precedence exists for these insertion-sequence-like DRs of *H. somnus* to play a role in the expression of the p76 gene.

No ATG codon in the sequence was an obvious translational start site, providing an appropriate length of ORF to give a theoretical 76 kDa protein. Thus, an attempt was made to find the start site for this gene by deletion analysis. Subclones of pHS138 were constructed (Fig. 4) and their cell lysates analysed for expression by Western blotting. The blots were incubated with P3 bovine convalescent phase serum, which previously was shown to react with the 270, 120 and 76 kDa surface proteins of *H. somnus* (Cole *et al.*, 1992; Corbeil *et al.*, 1988). The Western blots, also shown in Fig. 4, indicate that some inserts encoded proteins that react with P3 convalescent serum. The control cell lysate (*E. coli* DH5 α with pUC18 alone) did not react with the antiserum

under these conditions. In addition, a subclone (pHS145, with a 1 kb insert containing 0.5 kb of the 3' end of the ORF) encodes enough of the sequence to express a 23 kDa protein that reacts with P3 bovine convalescent antisera (data not shown). This suggests that at least one epitope on p76 that reacts with P3 antibody is within 167 amino acids from the C-terminus of the protein.

DR1 overlaps the putative translational initiation region of p76, since pHS138 expresses p76 and DR1 spans the *HpaI* site in pHS139 that was used to derive pHS138 (Figs 2 and 4). Also, each DR contains three ATG codons, which are repeated faithfully. All three of these ATG codons (labelled A, B and C in Figs 1 and 4) were candidates for start codons for synthesis of p76, as sufficient sequence remains in the ORF to encode a protein of that molecular mass. The starting plasmid, pHS138, contains all three of the ATG codons, and is not in frame with the *lacZ* protein to make a translational fusion. It expresses p76, plus some lower molecular mass antigens (weaker bands on the Western blot). Subclone pHS182 is missing codon A, but has B and C, and also is not in frame for a fusion with *LacZ*. However, this subclone does not express any antigens detected by the P3 antiserum. pHS176 contains the same insert as pHS182, but is in the proper frame with the *lacZ* start site to make a translational fusion protein. This clone expresses p76, plus some of the lower molecular mass antigens appearing as weaker bands on the Western blot. The insert which contains only start codon C, in pHS171, is not in frame to make a *lacZ* translational fusion protein, but does encode some of the same antigens below 76 kDa as pHS138. Plasmids pHS182, 176 and 171 were all deletion derivatives of pHS138, and thus all contained the *lacZ* promoter in the proper orientation to the *H. somnus* ORF. Since pHS138 expressed p76, and pHS182 does not, the start codon for p76 must lie between the *HpaI* site (contained in pHS138, but not 182) and the *PvuI* site (present in both plasmids). Since no other known start codons (TTG or GTG) are present within these two restriction sites, the start codon for p76 must be the ATG codon A. A possible ribosome binding site (AAAGAG) located 12 bp from the translational start may be functional in pHS139. This site occurs just upstream of the *HpaI* restriction site and would thus be deleted in pHS138, explaining the orientation-specific effect of the insert in this clone.

Since translation from codon A could theoretically produce a protein of 87 kDa, the protein must either be processed to form p76 or the protein runs anomalously on SDS-PAGE gels. An example of the former situation was found for the 120 kDa *Rickettsia rickettsii* OMP (Gilmore *et al.*, 1989, 1991), which contains enough DNA in the ORF to encode a deduced 168 kDa protein. Post-translational processing of the C-terminus appar-

ently gives rise to the 120 kDa product, since a 32 kDa peptide with the same sequence as the 3' end of the large ORF was found (Gilmore *et al.*, 1991). Interestingly, the 120 kDa ORF of *R. rickettsii* is also similar to *H. somnus* p76 since both contain only two cysteines (Gilmore *et al.*, 1989), providing minimal intramolecular disulphide bonding. The presence of only two cysteines in the *H. somnus* ORF explains the fact that no difference was observed (Cole *et al.*, 1992) in protein migration in SDS-PAGE gels run with and without urea, or with and without β -mercaptoethanol in the loading buffer. Those experiments, along with inclusion of protease inhibitors in the samples (Cole *et al.*, 1992), suggested that the multitude of bands observed on SDS-PAGE gels for recombinants containing the 76 kDa ORF was not due to artifacts such as monomer polymerization or *in vitro* proteolytic degradation of the *H. somnus* proteins.

The expression of lower molecular mass antigens at 66, 52 and 55 kDa by pHS171 which has codon C but not A or B, could be due to use of additional start codons. Codon C as a start site could produce a protein of theoretical molecular mass 52 kDa. The other two antigens may migrate anomalously on SDS-PAGE gels, or be modification (66 kDa) or degradation (50 kDa) products of the 52 kDa protein. These proteins were also expressed by pHS138. Expression from this alternative start site (codon C) within the 76 kDa ORF of lower molecular mass proteins could explain the multiple banding patterns on Western blots observed previously (Cole *et al.*, 1992). The use of more than one translational initiation site within an ORF has been described for numerous genes, including the *McrB* restriction system of *E. coli* (Ross *et al.*, 1989), as well as several phage (Dunn & Studier, 1983; Eisenberg & Finer, 1980; Fulford & Model, 1988) and transposon (Isberg *et al.*, 1982; Johnson & Reznikoff, 1984) genes. Expression of a *Mycoplasma hyorhinis* gene in *E. coli* resulted in the translation of related polypeptides of 110, 100, 65 and 55 kDa from an ORF of just over 3 kb (Notarnicola *et al.*, 1990). Tryptic peptide analysis revealed that these proteins all shared a common C-terminus, suggesting that all proteins were translated from a single message. A similar mechanism may occur for *H. somnus* p76 and lower molecular mass proteins.

In conclusion, the DRs and p76 DNA coding sequence lie within a 13.4 kb segment that is present in two virulent serum resistant strains, and absent in all four of the serum sensitive strains tested (Cole *et al.*, 1992). The 13.4 kb segment contains an ORF of at least 4.5 kb, with multiple start sites functional in *E. coli* and two 1.2 kb DRs. The coding sequence for p76 starts in DR1 and continues beyond DR2, which has flanking inverted repeats similar to insertion elements. The duplicated DRs are thus essential to the expression of a hydrophilic

76 kDa surface protein which is associated with serum resistance and virulence.

This work was supported, in part, by grant 89-37265-4648 from the United States Department of Agriculture.

References

- BAILIE, W. E., COLES, E. H. & WEIDE, K. D. (1973). Deoxyribonucleic acid characterization of a microorganism isolated from infectious thromboembolic meningoencephalomyelitis of cattle. *International Journal of Systematic Bacteriology* **23**, 231-237.
- BARBOUR, A. G., CARTER, C. J., BURMAN, N., FREITAG, C. S., GARON, C. F. & BERGSTROM, S. (1991). Tandem insertion sequence-like elements define the expression site for variable antigen genes of *Borrelia hermsii*. *Infection and Immunity* **59**, 390-397.
- COLE, S. P., GUINEY, D. G. & CORBEIL, L. B. (1992). Two linked genes for outer membrane proteins are absent in four non-disease strains of *Haemophilus somnus*. *Molecular Microbiology* **6**, 1895-1902.
- CORBEIL, L. B., BLAU, K., PRIEUR, D. J. & WARD, A. C. S. (1985). Serum susceptibility of *Haemophilus somnus* from bovine clinical cases and carriers. *Journal of Clinical Microbiology* **22**, 192-198.
- CORBEIL, L. B., CHIKAMI, G., YARNALL, M., SMITH, J. & GUINEY, D. G. (1988). Cloning and expression of genes encoding *Haemophilus somnus* antigens. *Infection and Immunity* **56**, 2736-2742.
- DUNN, J. J. & STUDIER, F. W. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *Journal of Molecular Biology* **166**, 477-535.
- EISENBERG, S. & FINER, M. (1980). Cleavage and circularization of single-stranded DNA: a novel enzymatic activity of ϕ X174 A* protein. *Nucleic Acids Research* **22**, 5305-5315.
- FULFORD, W. & MODEL, P. (1988). Regulation of bacteriophage ϕ 1 DNA replication. I. New functions for genes II and X. *Journal of Molecular Biology* **203**, 49-62.
- GILMORE, R. D., JR, JOSTE, N. & McDONALD, G. A. (1989). Cloning, expression and sequence analysis of the gene encoding the 120 kDa surface-exposed protein of *Rickettsia rickettsii*. *Molecular Microbiology* **3**, 1579-1586.
- GILMORE, R. D., JR, CIEPLAK, W., JR, POLICASTRO, P. F. & HACKSTADT, T. (1991). The 120 kilodalton outer membrane protein (rOmpB) of *Rickettsia rickettsii* is encoded by an unusually long open reading frame: evidence for protein processing from a large precursor. *Molecular Microbiology* **5**, 2361-2370.
- GOGOLEWSKI, R. P., KANIA, S. A., INZANA, T. J., WIDDERS, P. R., LIGGITT, H. D. & CORBEIL, L. B. (1987). Protective ability and specificity of convalescent serum from calves with *Haemophilus somnus* pneumonia. *Infection and Immunity* **55**, 1403-1411.
- GROOM, S. C., LITTLE, P. B. & ROSENDAL, S. (1988). Virulence differences among three strains of *Haemophilus somnus* following intratracheal inoculation of calves. *Canadian Journal of Veterinary Research* **52**, 349-354.
- HARRIS, F. W. & JANZEN, E. D. (1989). The *Haemophilus somnus* disease complex (Hemophilosis): a review. *Canadian Veterinary Journal* **30**, 816-820.
- HOLMES, D. S. & QUIGLEY, M. (1981). A rapid boiling method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114**, 193-197.
- HUMPHREY, J. D. (1982). *Haemophilus somnus*: colonization of the bovine reproductive tract. [As quoted in Groom *et al.* (1988) and Humphrey & Stephens (1983).] PhD thesis, University of Guelph, Ont., Canada.
- HUMPHREY, J. D. & STEPHENS, L. R. (1983). *Haemophilus somnus*: a review. *Veterinary Bulletin* **53**, 987-1004.
- INZANA, T. J. & CORBEIL, L. B. (1987). Further characterization of the growth requirements of *Haemophilus somnus*: development of a defined medium. *American Journal of Veterinary Research* **48**, 366-369.
- INZANA, T. J. & TODD, J. T. (1992). Immune response of cattle to *Haemophilus somnus* lipid A-protein conjugate vaccine and efficacy in a mouse abortion model. *American Journal of Veterinary Research* **53**, 175-179.
- ISBERG, R. R., LAAZAAR, A. L. & SYVANEN, M. (1982). Regulation of Tn5 by the right-repeat proteins: control at the level of the transposition reaction. *Cell* **30**, 883-892.
- JOHNSON, R. C. & REZNIKOFF, W. S. (1984). Role of the IS50R proteins in the promotion and control of Tn5 transposition. *Journal of Molecular Biology* **177**, 645-661.
- KWIECIEN, J. M. & LITTLE, P. B. (1991). *Haemophilus somnus* and reproductive disease in the cow: a review. *Canadian Veterinary Journal* **32**, 595-601.
- KYTE, J. & DOOLITTLE, R. F. (1982). A simple method for displaying the hydrophobic character of a protein. *Journal of Molecular Biology* **157**, 105-132.
- LO, R. Y. (1992). An analysis of the codon usage of *Pasteurella haemolytica* A1. *FEMS Microbiology Letters* **79**, 125-131.
- MILLS, J. A., VENKATESAN, M. M., BARON, L. S. & BUYSE, J. M. (1992). Spontaneous insertion of an IS1-like element into the *virF* gene is responsible for avirulence in opaque colonial variants of *Shigella flexneri* 2a. *Infection and Immunity* **60**, 175-182.
- NOTARNICOLA, S. M., MCINTOSH, M. A. & WISE, K. S. (1990). Multiple translational products from a *Mycoplasma hyorhinis* gene expressed in *Escherichia coli*. *Journal of Bacteriology* **172**, 2986-2995.
- PRITCHARD, A. E. & VASIL, M. L. (1990). Possible insertion sequences in a mosaic genome organization upstream of the exotoxin A gene in *Pseudomonas aeruginosa*. *Journal of Bacteriology* **172**, 2020-2028.
- ROSS, T. K., ACHBERGER, E. C. & BRAYMER, H. D. (1989). Nucleotide sequence of the McrB region of *Escherichia coli* K-12 and evidence for two independent translational initiation sites at the *mcrB* locus. *Journal of Bacteriology* **171**, 1974-1981.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SHARP, P. M., COWE, E., HIGGINS, D. G., SHIELDS, D. C., WOLFE, K. H. & WRIGHT, F. (1988). Codon usage patterns in *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster* and *Homo sapiens*; a review of the considerable within-species diversity. *Nucleic Acids Research* **16**, 8207-8211.
- THEISEN, M. & POTTER, A. A. (1992). Cloning, sequencing, expression, and functional studies of a 15000-molecular-weight *Haemophilus somnus* antigen similar to *Escherichia coli* ribosomal protein S9. *Journal of Bacteriology* **174**, 17-23.
- THEISEN, M., RIOUX, C. R. & POTTER, A. A. (1992). Molecular cloning, nucleotide sequence, and characterization of a 40000-molecular-weight lipoprotein of *Haemophilus somnus*. *Infection and Immunity* **60**, 826-831.
- VON HEIJNE, G. (1983). Patterns of amino acids near signal-sequence cleavage sites. *European Journal of Biochemistry* **133**, 17-21.
- WIDDERS, P. R., PAISLEY, L. G., GOGOLEWSKI, R. P., EVERMANN, J. F., SMITH, J. W. & CORBEIL, L. B. (1986). Experimental abortion and the systemic immune response in cattle to *Haemophilus somnus*. *Infection and Immunity* **54**, 555-560.
- WIDDERS, P. R., DORRANCE, L. A., YARNALL, M. & CORBEIL, L. B. (1989). Immunoglobulin-binding activity among pathogenic and carrier isolates of *Haemophilus somnus*. *Infection and Immunity* **57**, 639-642.
- YARNALL, M., GOGOLEWSKI, R. P. & CORBEIL, L. B. (1988a). Characterization of two *Haemophilus somnus* Fc receptors. *Journal of General Microbiology* **134**, 1993-1999.
- YARNALL, M., WIDDERS, P. R. & CORBEIL, L. B. (1988b). Isolation and characterization of Fc receptors from *Haemophilus somnus*. *Scandinavian Journal of Immunology* **28**, 129-137.